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(54) **METHOD FOR INCREASING EXPRESSION AND REDUCING EXPRESSION VARIABILITY OF FOREIGN GENES IN PLANT CELLS**

VERFAHREN ZUR ERHÖHUNG DER EXPRESSION UND ZUR VERMINDERUNG DER EXPRESSIONSVARIABILITÄT VON FREMDGENEN IN PFLANZENZELLEN

PROCEDE D'INTENSIFICATION DE DEGRES D'EXPRESSION ET DE REDUCTION DE LA VARIABILITE D'EXPRESSION DE GENES ETRANGERS DANS DES CELLULES DE VEGETAUX

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Description

Field of the Invention

[0001] The present invention relates to methods for reducing the variability of expression of foreign genes in plant cells, along with DNA constructs for carrying out such methods and the plant cells and plants so produced.

Background of the Invention

[0002] Agricultural biotechnology, and particularly plant biotechnology, has become recognized as one of the principal areas for the application of biotechnology techniques. Systems exist for transforming plant cells and regenerating complete plants from the transformed cells; structural gene and gene regulatory regions continue to be identified; and the need for plants with genetically engineered traits such as insect resistance and drought resistance remains strong.

[0003] A problem with the expression of foreign genes in plants is the clonal variation in the expression of the same gene in independent transformants: a problem referred to as "position effect" variation. No completely satisfactory method of obviating this problem has yet been developed, and there is accordingly a continued need for solutions to this problem.

[0004] Nuclear scaffold attachment regions (SARs) have been examined in tobacco plants (Molecular Biology of the Cell, Sept. 1992, Vol. 3, Abstract 776; Abstract of the Intl. Society for Plant Molecular Biology, 1991, Abstract 407). These SARs were found to increase levels of expression of transgenes when flaking the transgene in contrast to non-SAR constructs.

[0005] Meyer et al (Proc. Natl. Acad. Sci. USA, Vol. 85 pp 8568 - 8572, November 1988) describes the use of a genomic fragment isolated from petunia that increases hybridisation efficiencies. A fragment is identified as a "transformation booster sequence" and there is no disclosure of the use of such a booster sequence both 5' and 3' to a trans gene in this document.

[0006] WO 92/14822 describes a nucleic acid fragment useful in overexpressing a high methionine seed storage protein in plants. Although the use of suitable regulatory sequences operably connected to this seed storage protein DNA is described, the use of scaffold attachment regions is neither discussed or suggested.

[0007] Hoffman et al (EMBO Journal vol. 6 no. 11 pp. 3213 - 3221, 1987) describes the synthesis of a maize seed storage protein (zein) in transgenic tobacco seeds. Zein gene was placed under the regulation of bean 5' and 3' flanking regions. However, the use of SARS either 5' or 3' to the structural gene is not taught. Furthermore transformation was carried out using *Agro-bacterium*.

[0008] WO 93/19190 describes a nucleic acid fragment encoding aspartokinase, an enzyme that encourages the accumulation of certain amino acids in the seeds of plants. The use of SARS in combination with this structural gene is not described.

[0009] US 5,122,466 describes a method for transforming conifers with a DNA construct comprising an expression cassette. The use of scaffold attachment regions is neither disclosed or suggested in this document.

[0010] Breyn et al (Plant Cell, Vol. 4 pp 463 - 471, April 1992) isolated and characterised the nuclear scaffold from tobacco that binds specific plant DNA fragments in vitro. During investigation, scaffold attachment regions were used in conjunction with T-DNA vectors to investigate its functional rule in genic expression in vivo.

[0011] WO 91/13993 relates to seed specific expression cassettes for regulation and expression of proteins important for pharmacological and industrial needs in plants.

[0012] Thus, expression cassettes containing scaffold attachment regions have only been transfected into plant cells using *Agrobacterium* vector.

Summary of the Invention

[0013] In view of the foregoing, a first aspect of the present invention is a method of making recombinant plant cells having reduced variability of expression and increased levels of expression of foreign genes therein. The method comprises (a) providing a plant cell capable of regeneration; (b) transforming the plant cell with a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a transcription initiation region, a structural gene positioned downstream from the transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to the transcription initiation region or 3' to the structural gene, the expression cassette subject to the proviso that T-DNA borders are excluded therefrom and wherein expression of the structural gene is increased compared to that which would occur in the absence of said scaffold attachment regions. Preferably the transforming step is carried out by bombarding the plant cell with microparticles carrying the expression cassette. The transforming step is preferably followed by regenerating shoots, roots, or both shoots and roots (i.e., an intact plant) from the transformed cells. Preferably the DNA construct comprises, in the 5' to 3' direction, a first scaffold

attachment region, a transcription initiation region, a structural gene positioned downstream from the transcription initiation region and operatively associated therewith, a termination region, and a second scaffold attachment region.

[0014] A second aspect of the present invention is a DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a transcription initiation region, a structural gene positioned downstream from the transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to the transcription initiation region or 3' to the structural gene, the expression cassette subject to the proviso that T-DNA borders are excluded therefrom.

[0015] A third aspect of the present invention is a DNA construct as given above carried by a plant transformation vector, wherein the plant transformation vector is not *Agrobacterium tumefaciens.*

[0016] A fourth aspect of the present invention is a plant cell containing a DNA construct as given above.

[0017] A fifth aspect of the present invention is a recombinant plant comprising transformed plant cells, the transformed plant cells containing a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a transcription initiation region, a structural gene positioned downstream from the transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to the transcription initiation region or 3' to the structural gene, the expression cassette subject to the proviso that T-DNA borders are excluded therefrom.

[0018] The foregoing and other objects and aspects of this invention are explained in detail in the specification set forth below.

Brief Description of the Drawings

[0019] **Figure 1** schematically illustrated plasmids used to test the effect of flanking scaffold attachment regions on gene expression. Abbreviations: CaMV35S, cauliflower mosaic virus 35S promoter; β-glucuronidase, coding region of the *Escherichia coli* β-glucuronidase gene; NOS TERM, terminator from the nopaline synthase gene; SAR, scaffold attachment region from the yeast ARS-1 element; NOS, promoter from the nopaline synthase gene; OCS TERM, terminator from the octapine synthase gene.

Detailed Description of the Invention

[0020] The present invention may be carried out in a variety of plants (i.e., vascular plants) and the cells thereof to reduce expression variability therein, including both gymnosperms and angiosperms (i.e., monocots, dicots). Angiosperms are currently preferred.

[0021] The term "operatively associated", as used herein, refers to DNA sequences on a single DNA molecule which are associated so that the function of one is affected by the other. Thus, a transcription initiation region is operatively associated with a structural gene when it is capable of affecting the expression of that structural gene (i.e., the structural gene is under the transcriptional control of the transcription initiation region). The transcription initiation region is said to be "upstream" from the structural gene, which is in turn said to be "downstream" from the transcription initiation region.

[0022] DNA constructs, or "expression cassettes", of the present invention preferably include, 5' to 3' in the direction of transcription, a first scaffold attachment region, a transcription initiation region, a structural gene operatively associated with the transcription initiation region, a termination sequence including a stop signal for RNA polymerase and a polyadenylation signal for polyadenylation (e.g., the nos terminator), and a second scaffold attachment region, subject to the proviso that T-DNA borders are excluded from the DNA construct. All of these regions should be capable of operating in the cells of the tissue to be transformed. The termination region may be derived from the same gene as the transcriptional initiation or promoter region or may be derived from a different gene.

[0023] Scaffold attachment regions (or "SARs"), also called matrix attachment regions (or "MARs"), which are used to carry out the present invention may be of any suitable origin. In general, the SAR of any eukaryotic organism (including plants, animals, and yeast) may be employed, as SARs are highly conserved among the eukaryotes. *See, e. g.*, M. Eva Luderus et al., *Cell* **70**, 949 - 959 (1992); G. Hall et al., *Proc. Natl. Acad. Sci. USA* **88**, 9320 - 9324 (1991). For example, animal SARs are shown to be operational in plants in P. Breyne, *The Plant Cell* **4**, 463 - 471 (1992), and yeast SARs are shown to be operational in plants hereinbelow. Plant SARs may be taken from any suitable plant, including those plants specified above and below; animal SARs may be taken from any suitable animal including mammals (e.g., dog, cat), birds (e.g., chicken, turkey), etc.; and SARs may be taken from other eukaryotes such as fungi (e.g., *Saccharomyces cereviceae*). Where two scaffold attachment regions are employed, they may be the same or different. The length of the SAR is not critical so long as it retains operability as an SAR, with lengths of from 400 to 1000 base pairs being typical.

[0024] The transcription initiation region, which preferably includes the RNA polymerase binding site (promoter), may be native to the host plant to be transformed or may be derived from an alternative source, where the region is functional in the host. Other sources include the T-DNA genes from Agrobactrium (transformation is not performed using Agro-

bacterium), such as the transcriptional initiation regions for the biosynthesis of nopaline, octapine, mannopine, or other opine transcriptional initiation regions, transcriptional initiation regions from plants or woody species other than the host species, transcriptional initiation regions from viruses (including host specific viruses), or partially or wholly synthetic transcription initiation regions. Transcriptional initiation and termination regions are well known. *See, e.g.,* dGreve, *J. Mol. Appl. Genet.* **1**, 499 - 511 (1983); Salomon et al., *EMBO J.* **3**, 141 - 146 (1984); Garfinkel el al., *Cell* **27**, 143 - 153 (1983); and Barker et al., *Plant Mol. Biol.* **2**, 235 - 350 (1983).

[0025] The transcriptional initiation regions may not only include the RNA polymerase binding site, but may also include regions which regulate transcription, where the regulation involves, for example, chemical or physical repression or induction (e.g., regulation based on metabolites or light) or regulation based on cell differentiation, such as associated with leaves, roots, seed, or the like. Thus, the transcriptional initiation region, or the regulatory portion of such region, is obtained from an appropriate gene which is regulated, for example, the 1,5-ribulose biphosphate carboxylase gene, which is light-induced and used for transcriptional initiation, stress-induced genes, heat shock genes which are temperature regulated, wound induced genes, pathogen induced genes, meristem specific genes, genes of viruses specialized to function in plant cells, etc.

[0026] Structural genes are those portions of genes which comprise a DNA segment coding for a protein, polypeptide, or portion thereof, possibly including a ribosome binding site and/or a translational start codon, but lacking a transcription initiation region. The term can also refer to copies of a structural gene naturally found within a cell but artificially introduced. The structural gene may encode a protein not normally found in the plant cell in which the gene is introduced or in combination with the transcription initiation region to which it is operationally associated, in which case it is termed a heterologous structural gene. Genes which may be operationally associated with a transcription initiation region of the present invention for expression in a plant species may be derived from a chromosomal gene, cDNA, a synthetic gene, or combinations thereof. Any structural gene may be employed. The structural gene may encode an enzyme to introduce a desired trait into the plant, such as glyphosphate resistance; the structural gene may encode a protein such as a *Bacillus thuringiensis* protein (or fragment thereof) to impart insect resistance to the plant; the structural gene may encode a plant virus protein or fragment thereof to impart virus resistance to the plant.

[0027] The expression cassette may be provided in a DNA construct which also has at least one replication system. For convenience, it is common to have a replication system functional in *Escherichia coil,* such as ColE1, pSC101, pACYC184, or the like. In this manner, at each stage after each manipulation, the resulting construct may be cloned, sequenced, and the correctness of the manipulation determined. In addition, or in place of the *E. coli* replication system, a broad host range replication system may be employed, such as the replication systems of the P-1 incompatibility plasmids, e.g., pRK290. In addition to the replication system, there will frequently be at least one marker present, which may be useful in one or more hosts, or different markers for individual hosts. That is, one marker may be employed for selection in a prokaryotic host, while another marker may be employed for selection in a eukaryotic host, particularly the plant host. The markers may be protection against a biocide, such as antibiotics, toxins, heavy metals, or the like; provide complementation, by imparting prototrophy to an auxotrophic host: or provide a visible phenotype through the production of a novel compound in the plant. Exemplary genes which may be employed include neomycin phosphotransferase (NPTII), hygromycin phosphotransferase (HPT), chloramphenicol acetyltransferase (CAT), nitrilase, and the gentamicin resistance gene. For plant host selection, non-limiting examples of suitable markers are β-glucuronidase, providing indigo production, luciferase, providing visible light production, NPTII, providing kanamycin resistance or G418 resistance, HPT, providing hygromycin resistance, and the mutated aroA gene, providing glyphosate resistance.

[0028] The various fragments comprising the various constructs, expression cassettes, markers, and the like may be introduced consecutively by restriction enzyme cleavage of an appropriate replication system, and insertion of the particular construct or fragment into the available site. After ligation and cloning the DNA construct may be isolated for further manipulation. All of these techniques are amply exemplified in the literature and find particular exemplification in Sambrook et al., Molecular Cloning: A Laboratory Manual, (2d Ed. 1989) (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

[0029] Vectors which are used to transform plant tissue with DNA constructs of the present invention are non-*Agrobacterium* vectors, particularly ballistic vectors, as well as vectors suitable for DNA-mediated transformation.

[0030] Microparticles carrying a DNA construct of the present invention, which microparticles are suitable for the ballistic transformation of a plant cell, are also useful for making transformed plants of the present invention. The microparticle is propelled into a plant cell to produce a transformed plant cell, and a plant is regenerated from the transformed plant cell. Any suitable ballistic cell transformation methodology and apparatus can be used in practising the present invention. Exemplary apparatus and procedures are disclosed in Stomp et al., U.S. Patent No. 5,122,466; and Sandord and Wolf, U.S. Patent No. 4,945,050. When using ballistic transformation procedures, the expression cassette may be incorporated into a plasmid capable of replicating in the cell to be transformed. Examples of microparticles suitable for use in such systems include 1 to 5 μm gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

[0031] Plant species may be transformed with the DNA construct of the present invention by the DNA-mediated transformation of plant cell protoplasts and subsequent regeneration of the plant from the transformed protoplasts in accordance with procedures well known in the art.

[0032] Any plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a vector of the present invention. The term "organogenesis," as used herein, means a process by which shoots and roots are developed sequentially from meristematic centers; the term "embryogenesis," as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic cells or gametes. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristems, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

[0033] Plants of the present invention may take a variety of forms. The plants may be chimeras of transformed cells and non-transformed cells; the plants may be clonal transformants (e.g., all cells transformed to contain the expression cassette); the plants may comprise grafts of transformed and untransformed tissues (e.g., a transformed root stock grafted to an untransformed scion in citrus species). The transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, first generation (or T1) transformed plants may be selfed to give homozygous second generation (or T2) transformed plants, and the T2 plants further propagated through classical breeding techniques. A dominant selectable marker (such as *npt* II) can be associated with the expression cassette to assist in breeding.

[0034] Plants which may be employed in practicing the present invention include (but are not limited to) tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), soybean (*glycine max*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Ipomoea batatus*), cassava (*Manihot esculenta*), coffee (*Cofea* spp.), coconut (*Cocos nucifera*), pineapple (*Ananas comosus*), citrus trees (*Citrus* spp.), cocoa (*Theobroma cacao*), tea (*Camellia sinensis*), banana (*Musa* spp.), avocado (Persea *americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifera indica*), olive (*Olea* europaea), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia integrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), corn (*Zea mays*), wheat, oats, rye, barley, rice, vegetables, ornamentals, and conifers. Vegetables include tomatoes (*Lycopersicon esculentum*), lettuce (e.g., *Lactuea sativa*), green beans (*Phaseolus vulgaris*), lima beans (*Phaseolus limensis*), peas (*Pisum* spp.) and members of the genus *Cucumis* such as cucumber (*C. sativus*), cantaloupe (*C. cantalupensis*), and musk melon (*C. melo*). Ornamentals include azalea (*Rhododendron* spp.), hydrangea (*Macrophylla hydrangea*), hibiscus (*Hibiscus rosasanensis*), roses (*Rosa* spp.), tulips (*Tulipa* spp.), daffodils (*Narcissus* spp.), petnunias (*Petunia hybrida*), carnation (*dianthus caryophyllus*), poinsettia (*Euphorbia pulcherima*), and chyrsanthemum. Gymnosperms which may be employed to carrying out the present invention include conifers, including pines such as loblolly pine (*Pinus taeda*), slash pine (*Pinus elliotii*), ponderosa pine (*Pinus ponderosa*), lodgepole pine (*Pinus contorta*), and Monterey pine (*Pinus radiata*); Douglas-fir (*Pseudotsuga menziesii*); Western hemlock (*Tsuga canadensis*); Sitka spruce (*Picea glauca*); redwood (*Sequoia sempervirens*); true firs such as silver fir (*Abies amabilis*) and balsam fir (*Abies balsamea*); and cedars such as Western red cedar (*Thuja plicata*) and Alaska yellow-cedar (*Chamaecyparis nootkatensis*).

[0035] The examples which follow are set forth to illustrate the present invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Cell Maintenance for Bombardment

[0036] Suspension cultures of *Nicotinia Tabacum L*, line NT-1, were obtained from G. An, Washington State University. Cells were grown in a medium containing Murashige and Skoog salts (GIBCO Laboratories, Grand Island, NY), 100 mg/liter inositol, 1 mg/liter thiamine HCl, 180 mg/liter KH_2PO_4, 30 g/liter sucrose, and 2 mg/liter 2,4-dichlorophenoxyacetic acid. The pH of the medium was adjusted to 5.7 before autoclaving. The cells were subcultured once per week by adding 3 ml of inoculum to 100 ml of fresh medium in 500-ml Erlenmeyer flasks. The flasks were placed on a gyratory shaker at 125 rpm in a growth chamber adjusted to 27° C and constant light. Four day old cells, in early log phase, were used for bombardment.

[0037] Cells were prepared for bombardment by centrifuging 50-ml and resuspending the pellet to a concentration of 1g/ml which was subsequently diluted to 0.1g/ml with NT-1 broth. The diluted cells (0.5 ml) were spread as a monolayer onto lens paper on NT-1 agar (2% agar) support in 60 x 15 mm petri plates. These were kept at room temperature for three hours prior to bombardment.

EXAMPLE 2

Plasmid DNA and Microprojectile Coating

[0038] The β-glucuronidase (GUS) gene was used to measure expression and the neomycin phosphate transferase gene (nptII) was used for selection for stable transformation. The plasmids used in these transformation experiments are summarized in **Table 1** below. All plasmids were amplified in *Escherichia coli* strain DH5 alpha and were isolated by the Qiagen plasmid MAXIPREP™ kit. For each of the co-transformation plasmid mixtures, the molar ratio of GUS gene to nptII gene was 4:1. The DNA mixtures were associated with 1.0 um gold microprojectiles using $CaCl_2$/spermidine precipitation.

Table 1.

PLASMID SUMMARY.	
PLASMID	**DESCRIPTION**
pGA-1	The EcoR1 fragment containing the TRP/ARS-1 Scaffold Attachment Region of yRP7 (B. Amati and S. Gasser, Cell 54: 967-978 (1988)) was cloned into the unique EcoR1 site in the pJKK mf(1)(J. Kirschman and J. Cramer, Gene 68: 163-165 (1988)) vector polylinker.
pGCA-3	The HindIII fragment of pGA-1 containing the ARS-1 cloned into the unique HindIII site in the Bluescribe pBSM13(-) vector purchased from Stratagene.
pGCA8	Identical to pGCA3 except the EcoR1 sites have been destroyed with Mung Bean nuclease and religated.
pGCA6	EcoR1 fragment of WPF144 (from W. Fitzmaurice) containing the CaMV 35S promoter driving the dihydrofolate reductase (dhfr) gene with a nos terminator cloned into the unique EcoR1 site of pBI221.
pGCA12	Pst1/Kpn1 fragment of pGCA6 containing the CaMV 35S promoter driving the GUS gene with a nopaline synthase terminator and CaMV35S promoter driving the dhfr gene with a nos terminator cloned into the Pst1/Kpn1 site of the Bluscript II KS vector polylinker. Bluscript II was purchased from Stratagene. The BSSH2 site in the nos terminator of the GUS gene also has been destroyed with Mung Bean nuclease.
pGCA776	XbaI fragment of pGCA8 containing ARS-1 was cloned into the unique Spe site of pGCA12. This resulted in ARS-1 in a correct orientation at the 5' end of the fragment containing the CaMV35S promoter driving the GUS gene with a nos terminator and CaMV35S promoter driving the dhfr gene with a nos terminator.
pGCA887	HindIII/Sal 1 fragment of pGCA8 containing the ARS-1 cloned into the Hind III/Sal 1 site in the polylinker of the Bluescript pBC KS(+) vector purchased from Stratagene. The resulting plasmid has unique multiple cloning restriction sites flanking the ARS-1.
pBI221	The Hind III/EcoR1 fragment from pBI121 (R. Jefferson et al., EMBO J. 6: 3901-3907 (1987)) containing the CaMV35S promoter driving the GUS gene with a nos terminator was cloned into pUC19. This vector was purchased from Clontech. This expression plasmid is schematically illustrated in **Fig. 1A**.
PGCA905	Not I/EcoR1 fragment of pGCA776 containing the CaMV35S promoter driving the GUS gene with a nos terminator and CaMV35S promoter driving the dhfr gene with a nos terminator cloned into the NOT I/EcoR1 site in the pBC KS(+) vector purchased from Stratagene. The resulting plasmid has ARS-1 in correct orientation 5' of the GUS reporter gene. This expression plasmid was is schematically illustrated in **Fig. 1B**.
pGCA1055	EcoR1/SacII fragment of pGCA12 containing the CaMV35S promoter driving the GUS gene with a nos terminator cloned into the uniqu EcoR1/SacII of pGCA887. The resulting plasmid has ARS-1 in correct orientation 3' of the GUS reporter gene. This expression plasmid is schematically illustrated in **Fig. 1C**.
pGCA984	EcoR1/SacII fragment of pGCA776 containing the ARS-1 5' of the CaMV35S promoter driving the GUS gene with a nos terminator cloned into the unique EcoR1/SacII site of pGCA887. The resulting plasmid has ARS-1 in correct orientation flanking the GUS reporter gene. This expression plasmid is schematically illustrated in **Fig. 1D**.

Table 1. (continued)

PLASMID SUMMARY.	
PLASMID	**DESCRIPTION**
pUCNK1	This plasmid (L. Herrera-Estrella et al., in Plant Molecular Biology Manual B1: 1-22 (S. Gelvin and R. Schilperoot, Eds. 1988)) contains a nopaline synthase promoter (nos) driving neomycin phosphotransferase (nptII) with an octapine synthase terminator. Expression of this plasmid confers kanamycin resistance in plant cells. This selection plasmid is schematically illustrated in **Fig. 1E**.

EXAMPLE 3

Particle Accelerator

[0039] A DuPont PDS-1000 biolistic device was used in all microprojectile bombardments as described by the manufacturer. Briefly, the target cells were placed below the microprojectiles and the chamber was evacuated. The high pressure chamber was pressurized to 1×10^7 Pa (1500 psi) with helium gas which ruptures a disk. The resulting shock wave forces a Kapton disk coated with the microprojectiles onto a steel screen. The gold microprojectiles previously coated with DNA as described in Example 2 above continue onward to penetrate the NT-1 cells.

EXAMPLE 4

Recovery and Histochemical Screening of Stable Transformants

[0040] After bombardment, the petri plates were sealed with parafilm and incubated for 24 hours at 27° Centigrade under constant light. The lens paper was then carefully removed and transferred to fresh NT-1 agar plates containing 300 µg per ml kanamycin. Kanamycin resistant microcalli began to appear in approximately 3 weeks. The isolated microcalli were then transferred to fresh NT-1 agar containing 300 µg per ml kanamycin. Pieces of the microcalli were removed and placed into sterile microfuge tubes. The microcalli were then histochemically screened by adding 200 µL of 5-bromo-3-chloro-3-indolyl-β-D-glucuronic acid (X-gluc) and incubated for 24 hours at 37° Centigrade. Results (data not shown) indicated that the double SAR construct illustrated in **Fig. 1D** gives higher levels of gene expression when compared to the other constructs and increases the percentage or fraction of transformants with detectable expression of the GUS reporter gene. Both single SAR constructs (**Fig. 1B**; **Fig. 1C**) produced intermediate GUS expression levels, whereas when no SAR was present expression levels were extremely low.

[0041] The foregoing examples are illustrative of the present invention, and are not to be construed as limiting thereof.

Claims

1. A method of making recombinant plant cells having increased expression of foreign genes therein, said method comprising:

   providing a plant cell capable of regeneration;
   transforming said plant cell with a DNA construct comprising in the 5' to 3' direction, a transcription initiation region functional in plant cells, a structural gene positioned downstream from said transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to said transcription initiation region or 3' to said structural gene, said DNA construct subject to the proviso that T-DNA borders are excluded therefrom;

   wherein expression of the structural gene is increased compared to that which would occur in the absence of said scaffold attachment regions.

2. A method according to claim 1, which construct comprises, in the 5' to 3' direction, a first scaffold attachment region, a transcription initiation region, a structural gene positioned downstream from said transcription initiation region and operatively associated therewith, and a second scaffold attachment region.

3. A method according to claim 1, wherein said transforming step is carried out by bombarding said plant cell with microparticles carrying said expression cassette.

4. A method according to claim 1, wherein said plant cell resides in a plant tissue capable of regeneration.

5. A method according to claim 1, further comprising the step of regenerating shoots from said transformed plant cells.

6. A method according to claim 1, further comprising the step of regenerating roots from said transformed plant cells.

7. A method according to claim 1, further comprising the step of regenerating a plant from said transformed .plant cells.

8. A method according to claim 1, wherein said plant cells are monocot cells.

9. A method according to claim 1, wherein said plant cells are dicot cells.

10. A method according to claim 1, wherein said plant cells are gymnosperm plant cells.

11. A method according to claim 1, wherein said first and second scaffold attachment regions are yeast scaffold attachment regions.

12. A method according to claim 1, wherein said first and second scaffold attachment regions are plant scaffold attachment regions.

13. A DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a transcription initiation region, a structural gene positioned downstream from said transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to said transcription initiation region or 3' to said structural gene, said expression cassette subject to the proviso that T-DNA borders are excluded therefrom.

14. A DNA construct according to claim 13, which construct comprises, in the 5' to 3' direction, a first scaffold attachment region, a transcription initiation region, a structural gene positioned downstream from said transcription initiation region and operatively associated therewith, and a second scaffold attachment region.

15. A DNA construct according to claim 13 carried by a plant transformation vector.

16. A DNA construct according to claim 14, wherein said first and second scaffold attachment regions are yeast scaffold attachment regions.

17. A DNA construct according to claim 14, wherein said first and second scaffold attachment regions are plant scaffold attachment regions.

18. A plant cell containing a DNA construct according to claim 13.

19. A dicotyledonous plant cell containing a DNA construct according to claim 13.

20. A monocotyledonous plant cell containing a DNA construct according to claim 13.

21. A gymnosperm plant cell containing a DNA construct according to claim 13.

22. A recombinant plant comprising transformed plant cells, said transformed plant cells containing a heterologous DNA construct comprising an expression cassette, which construct comprises, in the 5' to 3' direction, a transcription initiation region, a structural gene position downstream from said transcription initiation region and operatively associated therewith, and a scaffold attachment region positioned either 5' to said transcription initiation region or 3' to said structural gene, said expression cassette subject to the proviso that T-DNA borders are excluded therefrom.

23. A recombinant plant according to claim 22, which construct comprises, in the 5' to 3' direction, a first scaffold attachment region, a transcription initiation region, a structural gene positioned downstream from said transcription initiation region and operatively associated therewith, and a second scaffold attachment region.

24. A recombinant plant according to claim 23, wherein said first and second scaffold attachment regions are yeast

scaffold attachment regions.

25. A recombinant plant according to claim 23, wherein said first and second scaffold attachment regions are plant scaffold attachment regions.

26. A recombinant plant according to claim 23, further comprising a termination sequence positioned downstream from said structural gene and operatively associated therewith, said termination sequence positioned 5' to said second scaffold attachment region.

27. A recombinant plant according to claim 22, which plant is a monocot.

28. A recombinant plant according to claim 22, which plant is a dicot.

29. A recombinant plant according to claim 22, which plant is a dicot selected from the group consisting of tobacco, potato, soybean, peanuts, cotton, and vegetable crops.

30. A recombinant plant according to claim 22, which plant is a gymnosperm.

Patentansprüche

1. Verfahren zum Herstellen von rekombinanten Pflanzenzellen mit erhöhter Expression von Fremdgenen darin, wobei das Verfahren umfaßt:

zur Verfügung stellen einer Pflanzenzelle, fähig zur Regenerierung,
Transformieren der Pflanzenzelle mit einer DNA Konstrukt, umfassend in der 5' bis 3' Richtung eine Transcriptionsinitiationsregion, funktionell in Pflanzenzellen, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden und eine Gerüstanheftungsregion, angeordnet entweder 5' zu der Transcriptionsinitiationsregion oder 3' zu dem Strukturgen, wobei das DNA Konstrukt unter dem Vorbehalt ist, daß T-DNA Grenzen davon ausgeschlossen sind,

wobei Expression des Strukturgens erhöht ist im Vergleich zu derjenigen, die in der Abwesenheit der Gerüstanheftungsregionen auftreten würde.

2. Verfahren nach Anspruch 1, wobei das Konstrukt umfaßt in der 5' bis 3' Richtung eine erste Gerüstanheftungsregion, eine Transcriptionsinitiationsregion, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden, und eine zweite Gerüstanheftungsregion.

3. Verfahren nach Anspruch 1, wobei die Transformationsstufe durchgeführt wird durch Bombardieren der Pflanzenzelle mit Mikroteilchen, die die Expressionskassette tragen.

4. Verfahren nach Anspruch 1, wobei die Pflanzenzelle auf einem Pflanzengewebe, wirksam zur Regenerierung, beruht.

5. Verfahren nach Anspruch 1, ferner umfassend die Stufe von Regenerieren von Sprößlingen aus den transformierten Pflanzenzellen.

6. Verfahren nach Anspruch 1, ferner umfassend die Stufe von Regenerieren von Wurzeln aus den transformierten Pflanzenzellen.

7. Verfahren nach Anspruch 1, ferner umfassend die Stufe von Regenerieren einer Pflanze aus den transformierten Pflanzenzellen.

8. Verfahren nach Anspruch 1, wobei die Pflanzenzellen Einkeimblattzellen sind.

9. Verfahren nach Anspruch 1, wobei die Pflanzenzellen Zweikeimblattzellen sind.

10. Verfahren nach Anspruch 1, wobei die Pflanzenzellen Gymnospermepflanzenzellen sind.

11. Verfahren nach Anspruch 1, wobei die ersten und zweiten Gerüstanheftungsregionen Hefegerüstanheftungsregionen sind.

12. Verfahren nach Anspruch 1, wobei die ersten und zweiten Gerüstanheftungsregionen Pflanzengerüstanheftungsregionen sind.

13. DNA Konstrukt, umfassend eine Expressionskassette, wobei das Konstrukt umfaßt in der 5' bis 3' Richtung eine Transcriptionsinitiationsregion, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden, und eine Gerüstanheftungsregion, angeordnet entweder 5' zu der Transcriptionsinitiationsregion oder 3' zu dem Strukturgen, wobei die Expressionskassette voraussetzt, daß T-DNA Grenzen davon ausgeschlossen sind.

14. DNA Konstrukt nach Anspruch 13, wobei das Konstrukt umfaßt in der 5' bis 3' Richtung eine erste Gerüstanheftungsregion, eine Transcriptionsinitiationsregion, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden, und eine zweite Gerüstanheftungsregion.

15. DNA Konstrukt nach Anspruch 13, getragen von einem Pflanzentransformationsvektor.

16. DNA Konstrukt nach Anspruch 14, wobei die ersten und zweiten Gerüstanheftungsregionen Hefegerüstanheftungsregionen sind.

17. DNA Konstrukt nach Anspruch 14, wobei die ersten und zweiten Gerüstanheftungsregionen Pflanzengerüstanheftungsregionen sind.

18. Pflanzenzelle, enthaltend ein DNA Konstrukt nach Anspruch 13.

19. Zweikeimblättrige Pflanzenzelle, enthaltend ein DNA Konstrukt nach Anspruch 13.

20. Einkeimblättrige Pflanzenzelle, enthaltend ein DNA Konstrukt nach Anspruch 13.

21. Gymnospermenpflanzenzelle, enthaltend ein DNA Konstrukt nach Anspruch 13.

22. Rekombinante Pflanze, umfassend transformierte Pflanzenzellen, wobei die transformierten Pflanzenzellen ein heterologes DNA Konstrukt, umfassend eine Expressionskassette, enthalten, wobei das Konstrukt umfaßt in der 5' bis 3' Richtung eine Transcriptionsinitiationsregion, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden, und eine Gerüstanheftungsregion, angeordnet entweder 5' zu der Transcriptionsinitiationsregion oder 3' zu dem Strukturgen, wobei die Expressionskassette voraussetzt, daß T-DNA Grenzen davon ausgeschlossen sind.

23. Rekombinante Pflanze nach Anspruch 22, wobei das Konstrukt umfaßt in der 5' bis 3' Richtung eine erste Gerüstanheftungsregion, eine Transcriptionsinitiationsregion, ein Strukturgen, angeordnet stromabwärts von der Transcriptionsinitiationsregion und wirksam damit verbunden, und eine zweite Gerüstanheftungsregion.

24. Rekombinante Pflanze nach Anspruch 23, wobei die ersten und zweiten Gerüstanheftungsregionen Hefegerüstanheftungsregionen sind.

25. Rekombinante Pflanze nach Anspruch 23, wobei die ersten und zweiten Gerüstanheftungsregionen Pflanzengerüstanheftungsregionen sind.

26. Rekombinante Pflanze nach Anspruch 23, ferner umfassend eine Terminationssequenz, angeordnet stromabwärts von dem Strukturgen und wirksam damit verbunden, wobei die Terminationssequenz 5' zu der zweiten Gerüstanheftungsregion angeordnet ist.

27. Rekombinante Pflanze nach Anspruch 22, wobei die Pflanze ein Einkeimblatt ist.

28. Rekombinante Pflanze nach Anspruch 22, wobei die Pflanze ein Zweikeimblatt ist.

29. Rekombinante Pflanze nach Anspruch 22, wobei die Pflanze ein Zweikeimblatt, ausgewählt aus der Gruppe, be-

stehend aus Tabak, Kartoffel, Sojabohne, Erdnüssen, Baumwolle und Pflanzenernten ist.

30. Rekombinante Pflanze nach Anspruch 22, wobei die Pflanze eine Gymnosperme ist.

Revendications

1. Procédé de fabrication de cellules de plante recombinantes ayant une expression accrue de gènes étrangers dans celles-ci, ledit procédé comprenant:

   la fourniture d'une cellule de plante apte à se régénérer;

   la transformation de ladite cellule de plante avec un produit d'assemblage d'ADN comprenant, dans la direction 5' vers 3', une région d'initiation de transcription fonctionnelle dans des cellules de plante, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une région de fixation de squelette placée soit en 5' par rapport à ladite région d'initiation de transcription soit en 3' par rapport audit gène structural, ledit produit d'assemblage d'ADN étant soumis à la condition selon laquelle les frontières d'ADN-T sont exclues de celui-ci;

   dans lequel l'expression du gène structural est accrue par rapport à celle qui se produirait en l'absence desdites régions de fixation de squelette.

2. Procédé selon la revendication 1, ledit produit d'assemblage comprenant, dans la direction 5' vers 3', une première région de fixation de squelette, une région d'initiation de transcription, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une seconde région de fixation de squelette.

3. Procédé selon la revendication 1, dans lequel ladite étape de transformation est effectuée en bombardant ladite cellule de plante avec des microparticules portant ladite cassette d'expression.

4. Procédé selon la revendication 1, dans lequel ladite cellule de plante se trouve dans un tissu de plante apte à se régénérer.

5. Procédé selon la revendication 1, comprenant en outre l'étape consistant à régénérer des pousses à partir desdites cellules de plante transformées.

6. Procédé selon la revendication 1, comprenant en outre l'étape consistant à régénérer des racines à partir desdites cellules de plante transformées.

7. Procédé selon la revendication 1, comprenant en outre l'étape consistant à régénérer une plante à partir desdites cellules de plante transformées.

8. Procédé selon la revendication 1, dans lequel lesdites cellules de plantes sont des cellules de monocotylédone.

9. Procédé selon la revendication 1, dans lequel lesdites cellules de plante sont des cellules de dicotylédone.

10. Procédé selon la revendication 1, dans lequel lesdites cellules de plante sont des cellules de plante gymnosperme.

11. Procédé selon la revendication 1, dans lequel lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de levure.

12. Procédé selon la revendication 1, dans lequel lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de plante.

13. Produit d'assemblage d'ADN comprenant une cassette d'expression, ledit produit d'assemblage comprenant, dans la direction 5' vers 3', une région d'initiation de transcription, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une région de fixation de squelette placée soit en 5' par rapport à ladite région d'initiation de transcription soit en 3' par rapport audit gène structural, ladite cassette

d'expression étant soumise à la condition selon laquelle les frontières d'ADN-T sont exclues de celle-ci.

14. Produit d'assemblage d'ADN selon la revendication 13, ledit produit d'assemblage comprenant, dans la direction 5' vers 3', une première région de fixation de squelette, une région d'initiation de transcription, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une seconde région de fixation de squelette.

15. Produit d'assemblage d'ADN selon la revendication 13, porté par un vecteur de transformation de plante.

16. Produit d'assemblage d'ADN selon la revendication 14, dans lequel lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de levure.

17. Produit d'assemblage d'ADN selon la revendication 14, dans lequel lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de plante.

18. Cellule de plante contenant un produit d'assemblage d'ADN selon la revendication 13.

19. Cellule de plante dicotylédone contenant un produit d'assemblage d'ADN selon la revendication 13.

20. Cellule de plante monocotylédone contenant un produit d'assemblage d'ADN selon la revendication 13.

21. Cellule de plante gymnosperme contenant un produit d'assemblage d'ADN selon la revendication 13.

22. Plante recombinante comprenant des cellules de plante transformées, lesdites cellules de plante transformées contenant un produit d'assemblage d'ADN hétérologue comprenant une cassette d'expression, ledit produit d'assemblage comprenant, dans la direction 5' vers 3', une région d'initiation de transcription, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une région de fixation de squelette placée soit en 5' par rapport à ladite région d'initiation de transcription soit en 3' par rapport audit gène structural, ladite cassette d'expression étant soumise à la condition selon laquelle les frontières d'ADN-T sont exclues de celle-ci.

23. Plante recombinante selon la revendication 22, ledit produit d'assemblage comprenant, dans la direction 5' vers 3', une première région de fixation de squelette, une région d'initiation de transcription, un gène structural positionné en aval de ladite région d'initiation de transcription et associé effectivement avec celle-ci, et une seconde région de fixation de squelette.

24. Plante recombinante selon la revendication 23, dans laquelle lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de levure.

25. Plante recombinante selon la revendication 23, dans laquelle lesdites première et seconde régions de fixation de squelette sont des régions de fixation de squelette de plante.

26. Plante recombinante selon la revendication 23, comprenant en outre une séquence de terminaison positionnée en aval dudit gène structural et associée effectivement avec celui-ci, ladite séquence de terminaison étant positionnée en 5' par rapport à ladite seconde région de fixation de squelette.

27. Plante recombinante selon la revendication 22, ladite plante étant une monocotylédone.

28. Plante recombinante selon la revendication 22, ladite plante étant une dicotylédone.

29. Plante recombinante selon la revendication 22, ladite plante étant une dicotylédone choisie dans le groupe constitué par le tabac, la pomme de terre, le soja, l'arachide, le coton et les cultures légumières.

30. Plante recombinante selon la revendication 22, ladite plante étant un gymnosperme.

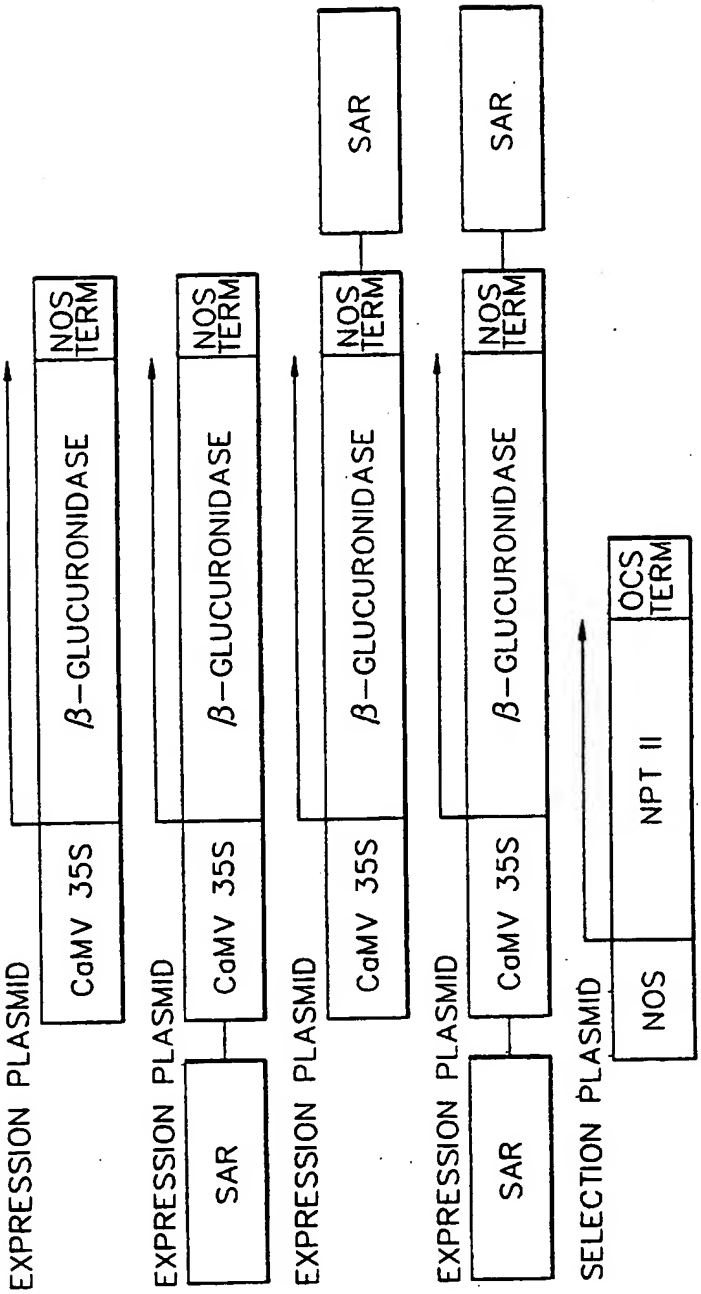

FIG. 1A.

FIG. 1B.

FIG. 1C.

FIG. 1D.

FIG. 1E.